

Preliminary Amendment

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Applicant(s): Timothy E. Benson

Serial No.: 09/772,598

Confirmation No.: 2967

Filed: January 30, 2001

For: CRYSTALLIZATION AND STRUCTURE DETERMINATION OF STAPHYLOCOCCUS AUREUS NAD SYNTHETASE

Please replace the paragraph at page 34, lines 13-21, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

S. aureus NadE binding compounds may be designed "de novo" using either an empty binding site or optionally including some portion(s) of a known inhibitor(s). There are many de novo ligand design methods including, without limitation, LUDI (H.-J. Bohm, J. Comp. Aid. Molec. Design 6:61-78 (1992); available from Molecular Simulations Inc., San Diego, CA); LEGEND (Y. Nishibata et al., Tetrahedron, 47:8985-8990 (1991); available from Molecular Simulations Inc., San Diego, CA); LeapFrog (available from Tripos Associates, St. Louis, MO); and SPROUT (V. Gillet et al., J. Comput. Aided Mol. Design 7:127-153 (1993); available from the University of Leeds, UK).

Please replace the paragraph at page 40, line 20 to page 41, line 9 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

The purified protein was determined to be >96% pure by SDS-PAGE. The sample was received in 25mM Tris, pH 8.0, 5mM β -mercaptoethanol (BME) as requested. BME was added to prevent loss of activity during storage (Zalkin, Methods Enzymol., 113:297-302 (1985)). This buffer system is amenable to crystallization, therefore no buffer exchanges were necessary. The protein was directly concentrated to approx. 20 mg/mL using a pretreated Ultrafree-4 10,000 MWCO concentrator (Millipore). Concentration determination was done by concentration factors based on the original Bradford assay results. Amino acid analysis indicated that the concentration was actually 15 mg/mL, this is a 24% decrease from the earlier calculation. Freshly prepared

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sample was split into 50 μ L aliquots, flash frozen in liquid nitrogen, and stored at -80°C . NAD synthetase was screened using the hanging drop method, in 24 well VDX plates (Hampton Research, Laguna Niguel, CA). The crystallization library consists of Hampton Research Crystal Screen I, Crystal Screen II, and Crystal Screen I- Lite (all available from Hampton Research, Laguna Niguel, CA) and Wizard I, Wizard II, Cryo I, and Cryo II (all available from Emerald Biostructures, Inc., Bainbridge Island, WA). NAD synthetase was screened in all conditions, with hits in Hampton Crystal Screen 1-Lite/43 (15% PEG 1500), Hampton Crystal Screen 1/39 (2% PEG 400, 2.0 M Ammonium Sulfate, 0.1 M Na HEPES pH 7.5), and Wizard 1/41 (30% PEG-3000, CHES pH 9.5).

Please replace the paragraph at page 43, lines 14-23 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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Because these data were of higher quality than the multiple anomalous dispersion data, a molecular replacement solution was initially attempted using the NadE dimer from *Bacillus subtilis* (1nsy.pdb (Rizzi et al., EMBO J., 15:5125-34 (1996))). A rotation solution was found using AMORE (Navaza, Acta Cryst., A50:157-63 (1994)) with a peak height of 8.6σ and a subsequent translation solution with a correlation coefficient of 26.9 and an R-factor of 50.3%. Subsequent refinement and rebuilding of this model (and other molecular replacement solutions from X-PLOR rotation/translation searches) led to an R-factor of 40% with a Free R-factor above 50%. Since this solution was difficult to refine, further efforts for a structure solution were attempted using the multiple anomalous dispersion data.

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Please replace the paragraph at page 45, lines 10-17 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

a5
Molecular replacement experiments were conducted with either AMORE (Navaza, Acta Cryst., A50:157-63 (1994)) or X-PLOR using the *B. subtilis* model of NAD synthetase (PDB id code 1nsy). A portion of the model (residues 106-125) was truncated where the identity of the two proteins was significantly lower than the remainder of the sequence. Using this truncated model, the initial solution for the first dimer gave a correlation coefficient of 18.8 with an R-factor of 51.2%. Searching for the second dimer led to an improved correlation coefficient of 30.0 with an R-factor of 48.5%.

Please replace the paragraph at page 48, line 14 to page 49, line 2 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

a6
Model building was done using the program CHAIN (Sack, J. Molecular Graphics, 6:224-25 (1988)) and LORE (Finzel, Meth. Enzymol., 277:230-42 (1997)). Model for one dimer built using the *B. subtilis* NAD synthetase structure as a reference. The second dimer was placed using non-crystallographic translational symmetry from the molecular replacement solution (R-factor/Free R-factor = 34.1%/39.9%), and refined using positional refinement, torsion angle dynamics and individual B-factor refinement (R-factor/Free R-factor = 24.9%/34.7%). At this stage waters were added and each monomer was thoroughly checked against the electron density. A further rounds of refinement led to the present model (R-factor/Free R-factor = 22.6%/31.2%). All refinement

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ab cycles were carried out with XPLOR98 (Brunger, X-PLOR version 3.1, Yale University Press (1992)) incorporating bulk solvent correction during the refinement (Jiang et al., J. Mol. Biol., 243:100-15 (1994)). Progress of the refinement was monitored by a decrease in both the R-factor and Free R-factor. Stereochemistry of the model was checked using PROCHECK (Laskowski et al, J. Appl. Cryst., 26:283-91 (1993)) revealing no residues in disallowed regions of the Ramachandran plot. Figure 5 was produced in MOLSCRIPT (Kraulis, J. Appl. Cryst., 24:946-50 (1991)) and Raster 3D (Merritt et al., Acta Cryst., D50:869-73 (1994) while Figures 6-8 were produced in MOLSCRIPT (Kraulis, J. Appl. Cryst., 24:946-50 (1991)) alone. Figures 11 and 12 were created in Mosaic-2.
